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Kinetics of β-cyclodextrin production by cyclodextrin glucanotransferase from *Bacillus megaterium*

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The kinetics of β-cyclodextrin production by cyclodextrin glucanotransferase from *Bacillus megaterium* was investigated using two different approaches. By studying the initial rate of the enzyme reaction a strong inhibition of CGTase by the main product of the cyclization reaction β-CD was revealed. The type of inhibition was determined to be mixed competitive and uncompetitive. The value of the uncompetitive inhibition constant (K_{iu}=33.30 mg/mL) was much higher than the value of the competitive inhibition constant (K_{ic}=4.21 mg/mL), which indicated a predominance of the competitive part of the mixed type inhibition. The dynamics of β-CD formation and the corresponding starch degradation was investigated at real process conditions with different initial substrate concentrations. The enzyme reaction rate was maximal at the beginning of the process when the substrate current concentration was maximal. Depletion of the substrate led to a corresponding decrease in rate values. Another reason for the decrease in the enzyme rate was the accumulation of the product β-CD, which inhibited CGTase. The results for the dynamics of beta CD production were used for investigation of the kinetics of the process at real process conditions. In a real CD production process, CGTase showed a change in the type of inhibition by β-CD. The type of inhibition was determined to be competitive, with inhibition constant Ki=6.13 mg/mL. A probable reason for the change of the type of inhibition at real process conditions was the change in the substrate and product concentrations during the process.

Keywords: cyclodextrin glucanotransferase**,** cyclodextrins, enzyme kinetics, product inhibition, *Bacillus megaterium*

INTRODUCTION

Cyclodextrins (CD) are non-reducing cyclic oligosaccharides composed of 6, 7 or 8 α-1,4-linked glucose residues, denoted as α, β and γ, respectively. They are obtained under the catalytic action of cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19). CGTase is a glycosyltransferase enzyme that displays four catalytic activities, named cyclization, coupling, disproportionation and hydrolysis [1, 2]. CD are formed by the cyclization activity of the enzyme. The cyclization activity of CGTase is an intramolecular transglycosylation reaction of long linear maltooligosaccharide chains, in which the cyclic ring structure of CD is formed [3]. CGTase can produce CD from substrates containing α-1,4-glycosyl chain, such as starch, dextrins, amylose, amylopectin, glycogen. The most widely used raw material for CD production is starch.

The processes for CD production described in the literature differ significantly in the yield of final products and the ratio between different CD types. The yield of CD varies from 15 to 93%, and the ratio α:β:γ - from the complete predominance of one type of CD to a mixture of all three types. Both the yield and the ratio of CD depend on the properties of CGTase, the type of substrate, its pre-treatment, the reaction conditions (substrate concentration, enzyme dosage, pH, temperature, duration of the process), the presence of organic compounds, etc. [4 – 7].

Despite of the selection of optimal reaction conditions, a complete conversion of the substrate to CD is not feasible. The main limiting factor is the product inhibition of CGTase by CD [8 – 11].

According to the literature, many CGTases are inhibited by the products of the cyclization reaction. The type of CD (α, β or γ) strongly inhibits the corresponding cyclization activity of the enzyme [12 –16]. Product inhibition of CGTase adversely affects the production of CD, as it leads to a reduction in the rate of product accumulation and/or to a complete cessation of the reaction, resulting in low CD yield.

Despite numerous reports of product inhibition of CGTase by CD, there are few explanations for this feature of the enzyme. According to Penninga *et al.* [17] CD bind to two parts of the enzyme molecule - the active site, which leads to competitive inhibition,

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and to the maltose-binding site in domain E, which disrupts its functions. Determination of the mechanism of inhibition is possible by studying the kinetics of the process. This also allows determination of the kinetic constants that have important technological meaning for the efficiency of the process.

Two approaches can be used to study product inhibition - experimental determination of the initial rate with the addition of different inhibitor concentrations [18] or determination of the rate during the process without additional introduction of inhibitor by studying the progress curves [19, 20]. The advantage of the second method is the obtaining of results that actually describe the process.

The aim of the present work is to study the kinetics of β-CD production by CGTase from *Bacillus megaterium* and to determine the type of inhibition and the values of the kinetic constants.

EXPERIMENTAL

CGTase enzyme preparation

Crude CGTase enzyme produced by *Bacillus megaterium* was used for the kinetics studies. The enzyme was a concentrate with an activity of 1.61E/mL. Enzyme production was described in a previous research [6, 21].

Determination of product inhibition of CGTase by β-CD

Product inhibition of CGTase by β-CD was studied by determination of the initial rate of the enzyme reaction at different concentrations of added inhibitor. Corn starch () was used as a substrate for cyclization reaction. The initial substrate concentration was in the range of 10-100 mg/mL, and the added product inhibitor β-CD varied in the range of 1-6 mg/mL. Corn starch suspensions with the desired concentrations were prepared in phosphate buffer with pH 7.0 by boiling in water bath for 10 min, the corresponding β-CD concentration and CGTase in the dosage of 2 U/g starch were added. The cyclization reaction was performed at 45°C for 10 min, the enzyme was inactivated by 10 min boiling and the concentration of β-CD formed was determined. The initial rate of the reaction was defined as the concentration of β-CD formed by the enzyme for 1 min under the assay conditions. The type of inhibition was determined by applying the Lineweaver–Burk transformation [18], and the kinetic constants K_m, V_m, and the inhibition constants K_{ic} and K_{iu} were calculated by using the model of mixed type inhibition:

$$v = \frac{V_m.S}{(K_m.(1+\frac{I}{Kic}) + S.(1+\frac{I}{Kiu}))} \quad (1)$$

v - initial rate of enzyme reaction, mg/(mL.min);

V_m – maximum rate of enzyme reaction, mg/(mL.min);

S – starch concentration, mg/mL;

K_m – Michaelis-Menten constant, mg/mL;

I – Inhibitor concentration, mg/ml;

K_{ic} – Competitive inhibition constant, mg/mL;

K_{iu} – Uncompetitive inhibition constant, mg/mL.

Investigation of the dynamics of β-CD production

The dynamics of β-CD production was studied at different corn starch () concentrations in the range of 10-100 mg/mL. Starch suspensions were prepared in phosphate buffer with pH 7.0 and heated in a boiling water bath for 10 min, cooled to 45°C and CGTase was added in a dosage of 2 U/g starch. The enzyme reaction was carried out at 45 °C with constant stirring for up to 7 h. Samples were taken at regular intervals, the enzyme was inactivated by boiling in a water bath for 10 min and the content of β-CD in the reaction mixture was determined.

Investigation of the kinetics of β-CD production by the progress curves of the process

The kinetics of the process of β-CD production was studied by the rate of product formation, defined as:

$$v = \frac{dP}{dt} \quad (2)$$

v – rate of enzyme reaction, mg/(mL.h);

P - concentration of β-CD, mg/mL;

t – time, h.

For determination of the type of inhibition and the kinetic constants reversible competitive and uncompetitive inhibition models were used [20]. The models for competitive and uncompetitive inhibition have the following type, respectively:

$$v = \frac{V_m.S}{K_m(1+\frac{I}{K_I}) + S} \quad (3)$$

$$v = \frac{V_m.S}{(K_m + S).(1+\frac{I}{K_I})} \quad (4)$$

v – rate of enzyme reaction, mg/(mL.h);

V_m – maximum rate of enzyme reaction, mg/(mL.h);

S – starch concentration, mg/mL;

K_m – Michaelis-Menten constant, mg/mL;

K_I – Inhibition constant, mg/mL.

The computing procedures were performed by using a package of applied software programs of own development in the software media of Matlab and Eureka [22]. The kinetic parameters for both models were calculated according to the optimization procedure minimizing the following criterion:

$$J = \sum_{i=1}^{n} (v_{\exp} - v_{\mathrm{mod}})^2 \rightarrow \min \qquad (5)$$

v_{exp} – experimental values of enzyme reaction rate, mg/(mL.h);

v_{mod} – predicted values of enzyme reaction rate, mg/(mL.h);

n – number of analyzed points.

Assays

The concentration of β-CD was determined by the spectrophotometric method with phenolphthalein. A freshly prepared solution of 1 part of 3.8 mM phenolphthalein in 95 % ethanol and 50 parts of carbonate buffer (pH=10.5) was prepared. Appropriately diluted samples of the reaction mixture were mixed with 2.0 mL of the phenolphthalein solution and distilled water to a final volume of 5.0 mL. The absorbance was measured at 550 nm against a blank sample, containing a mixture of water and phenolphthalein. β-Cyclodextrin concentration was calculated using a calibration curve. Starch concentration was determined by the method described by Thiemann *et al.* [23].

RESULTS AND DISCUSSION

Product inhibition of CGTase by β-CD

Most known CGTases are reported to be characterized with strong product inhibition by the predominant type of CD [12 – 14]. The investigated CGTase produced the three types of CD, and the predominant type was β-CD independently of the reaction conditions [11]. In order to determine the type of inhibition of the enzyme by β-CD, the kinetics of the process was studied. The initial rate of β-CD formation (v) was investigated at starch concentrations (S) in the range of 10-100 mg/mL with addition of 1-6 mg/mL inhibitor β-CD. The results are plotted in accordance with Lineweaver–Burk transformation in Fig. 1.

The results confirmed that CGTase from *B. megaterium* was strongly inhibited by the main product of the cyclization reaction. The studying of the Lineweaver–Burk plot (Fig. 1.) revealed a mixed type of inhibition of CGTase by β-CD. In the presence of β-CD both kinetic constants K_m and V_m were changing, and the inhibition was actually combined competitive and uncompetitive.

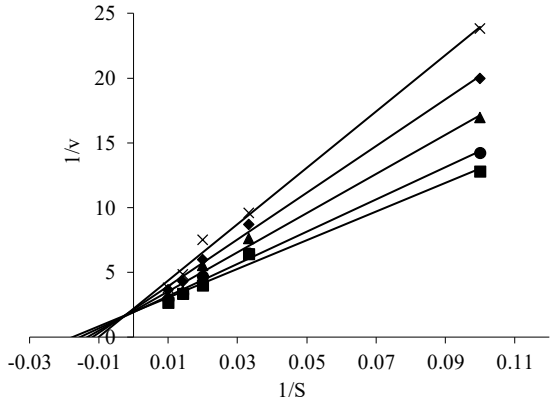

Fig. 1. Lineweaver–Burk plot of product inhibition of CGTase by different concentrations of β-CD: ■ 1 mg/mL, ● 2 mg/mL ▲ 3 mg/mL ◆ 5 mg/mL × 6 mg/mL.

The values of the kinetic and inhibition constants for mixed type inhibition in the presence of β-CD were calculated (Table 1). It was noted that the value of the uncompetitive inhibition constant (K_{iu}) was much higher than the value of the competitive inhibition constant (K_{ic}). This indicated a predominance of the competitive part of the mixed type inhibition. A mixed type of inhibition was also proved for other CGTases predominantly producing β-CD [12], while competitive inhibition was observed for enzymes forming mainly α-CD [12, 24].

Table 1. Kinetic and inhibition constants of CGTase for mixed type inhibition by β-CD

K_m, mg/mL	V_m, mg/(mL.min)	K_{ic}, mg/mL	K_{iu}, mg/mL
46.93	0.54	4.21	33.30

Dynamics of β-CD formation

The established mixed type of product inhibition by β-CD is an important characteristic of CGTase but it refers to the initial rate of the enzyme reaction at certain reaction conditions. It does not actually describe the process of β-CD production, which can be performed at different reaction conditions, with long duration of the process and at different actual concentrations of the product inhibitor. In order to study the product inhibition of CGTase by β-CD at real process conditions, the dynamics of β-CD formation and starch degradation were investigated at five different initial substrate concentrations. The results are presented in Fig. 2 A-E.

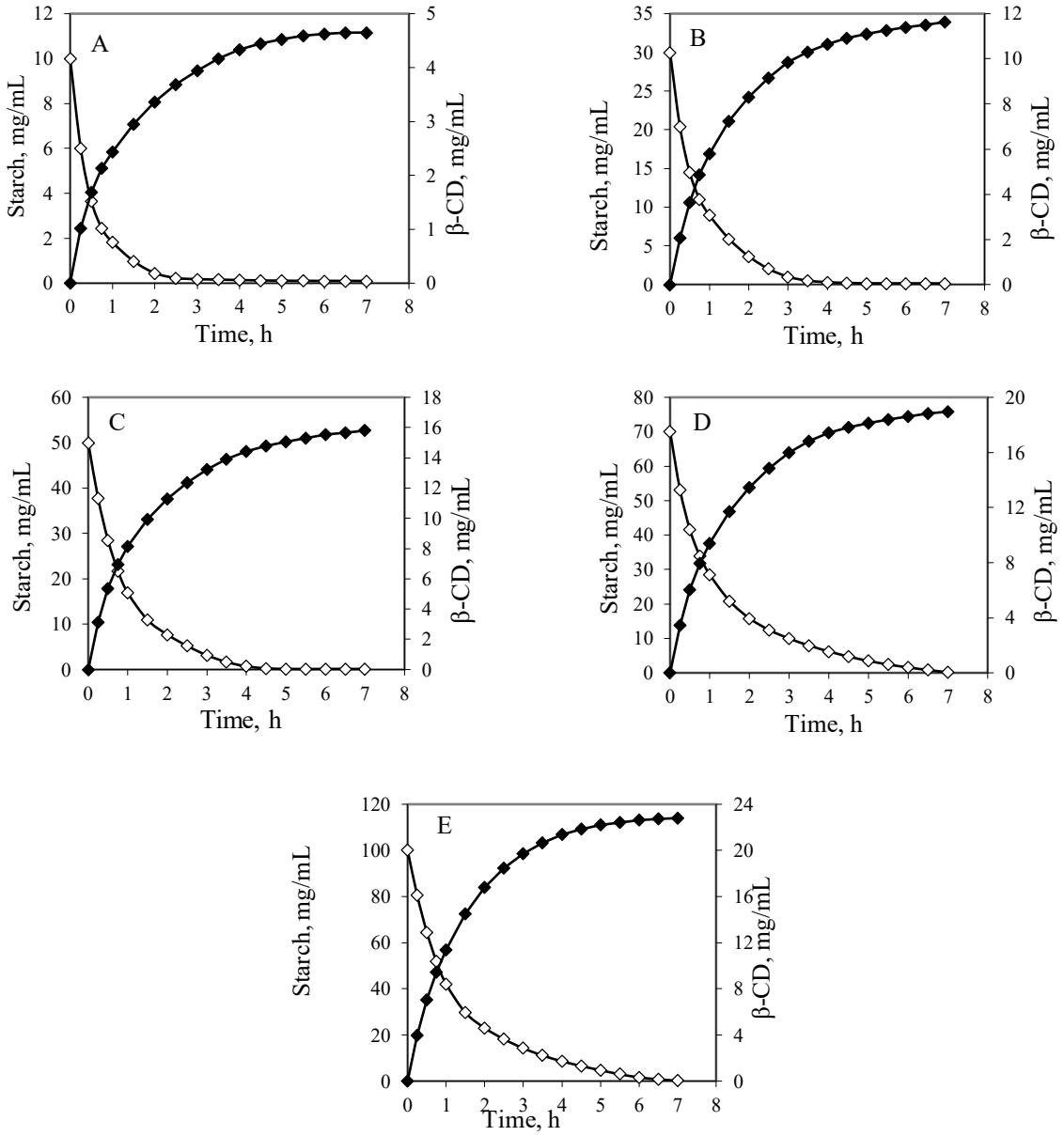

Fig. 2. Dynamics of β-CD formation (◆ β-CD) and starch degradation (◇ Starch) at different initial starch concentrations: A-10 mg/mL starch; B-30 mg/mL starch; C-50 mg/mL starch; D-70 mg/mL starch; E-100 mg/mL starch

For all studied initial substrate concentrations, the enzyme reaction started at high speed. The main amount of product was accumulated in the first 4 hours of the process. The rapid formation of β-CD corresponded to the rapid degradation of the substrate. At a starch concentration of 50 mg/mL, 50 % of the available substrate was depleted in the first 30 min.

The higher starch concentration required longer enzyme action. In this case, however, a reaction time of 5 hours was sufficient to form most of the product and thus to degrade most part of the substrate.

Kinetics of β-CD production by the progress curves of the process

In Fig. 3 the calculated rate values of the enzyme reaction are presented as a function of the current substrate concentration during the process. The results for the respective product concentration are also included.

The enzyme reaction rate was maximal at the beginning of the process when the substrate current concentration was maximal. The highest rate was reached at 100 mg/mL of starch. Depletion of the substrate led to a corresponding decrease in rate values. When comparing the results of Fig. 3 A-E it

was clear that substrate depletion was not the only reason for rate reduction. Provided the same amount of starch, the reaction rate depended on the concentration of β-CD in the reaction medium. This is illustrated in Fig. 4, where the rate of the process is represented as a function of the concentration of β-CD at the same current starch concentration.

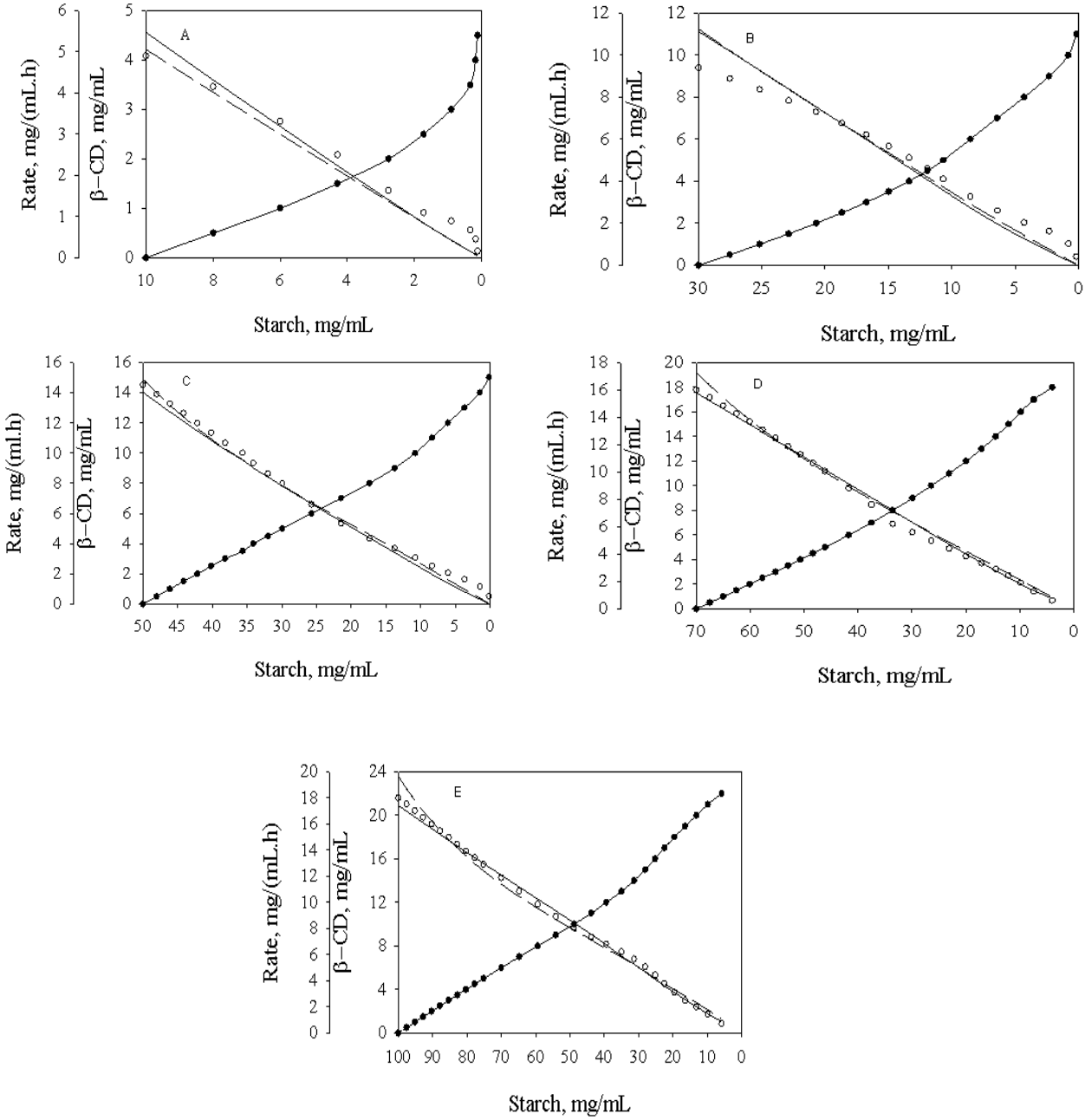

Fig. 3. Dependence of the enzyme reaction rate on current substrate concentration: ● experimental β-CD values; ○ experimental enzyme reaction rate; ─ model values for competitive inhibition; - - model values for uncompetitive inhibition at different initial starch concentrations: A-10 mg/mL starch; B-30 mg/mL starch; C-50 mg/mL starch; D-70 mg/mL starch; E-100 mg/mL starch.

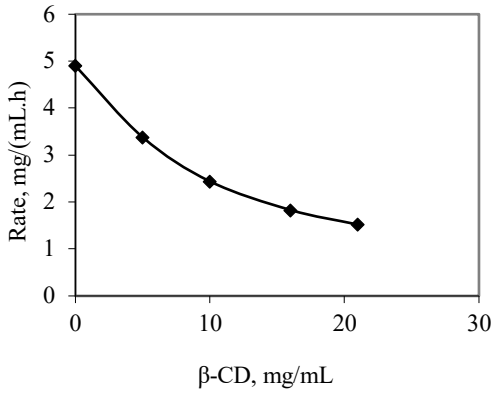

Fig. 4. Inhibition of CGTase by β-CD at current starch concentration of 10 mg/mL

The results showed that the increase in the concentration of the product led to a significant decrease in enzyme reaction rate. Even in the presence of only 5 mg/mL of β-CD, the process rate decreased by about 30%, and at 10 mg/mL CGTase synthesized β-CD at a twice lower rate. This fact is an indication of the strong effect of β-CD concentration on β-CD production process and it is in accordance with the established product inhibition of CGTase.

In order to determine the type of inhibition of the β-CD production process in real process conditions, the results of Fig. 3 A-E were processed according to models for mixed type (1), competitive (3) and uncompetitive inhibition (4). Though CGTase showed mixed type inhibition by β-CD by studying the initial rate of the enzyme reaction, this was not proved for real process conditions. The experimental data did not fit the model for mixed type inhibition (data not shown). For these reasons models for competitive (3) and uncompetitive inhibition (4) were evaluated. The calculated model values of rate and product concentration by the two models studied are presented in Fig. 3 A-E with lines. The variance of the models from the experimental values was used to evaluate the obtained models. The values of the kinetic parameters determined by equations (3) and (4) are presented in Table 2.

Table 2. Kinetic and inhibition constants of β-CD production

Constants	Competitive inhibition	Uncompetitive inhibition
V_m, mg/(mL.h)	23.01	29.03
K_m, mg/mL	32.02	47.43
K_i, mg/mL	6.13	11.53

The competitive inhibition model better described the process of β-CD production. This result is explainable given the mechanism of action of CGTase. β-CD compete with starch for a place in the active site and under appropriate conditions CGTase exhibits decyclization activity. The larger deviations of the models from the experimental results are observed at low initial starch concentration (Fig. 3 A, B). We hypothesize that this was due to the low concentration of β-CD and the limitation by substrate.

CONCLUSIONS

CGTase from *B. megaterium* was strongly inhibited by the main product of the cyclization reaction β-CD. By studying the initial rate of the enzyme reaction the type of inhibition was proved to be combined competitive and uncompetitive. The value of the uncompetitive inhibition constant (K_{iu}=33.30 mg/mL) was much higher than the value of the competitive inhibition constant (K_{ic}=4.21 mg/mL). This indicated a predominance of the competitive part of the mixed type inhibition.

At real process conditions, CGTase did not show a mixed type inhibition by β-CD. The process of β-CD production was influenced by competitive inhibition of the enzyme by β-CD, with inhibition constant Ki=6.13 mg/mL. A probable reason for the change of the type of inhibition at real process conditions was that the concentration of the product increased during the process and reached values much higher than the values available at the initial reaction time. At the same time, at long durations of the process, the substrate was depleted and reached minimal levels. This obviously led to a change of the type of inhibition from mixed to competitive, as beta CD compete with the substrate for the active sites of the enzyme.

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